

BACTERIA IN A PINE FOREST SOIL

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INTRODUCTION

Studies concerning the ecology of soil bacteria and the biochemical processes which occur in soil must be based upon a knowledge of the main types of bacteria which live and grow in this habitat. Because of the enormous spatial and temporal variations in the numbers and types of organisms present in a soil, a complete characterization of the bacterial flora is not yet feasible. Indeed, it may never become feasible. It is possible, however, to characterize the population at one or a few points in space and time and to study factors affecting the distribution of the bacteria which have been shown to be present under such restricted conditions.

To carry out such work it is essential to use techniques which permit the isolation of either a large random sample of the total microflora or of a segment of it in which one is interested. Secondly it is necessary to characterize as many of these organisms as possible on the basis of a varied and comprehensive range of properties (Gray and Goodfellow, 1966). Such work has been started on the microflora of a developing podzol soil which has formed under a cover of *Pinus nigra laricio*. This investigation has been concerned initially with determining the principal types of both fungi (Indraratne, 1964) and bacteria present. This paper reports the preliminary results concerning the bacteria.

The soil being studied occurs in a small area of pine forest planted on sand dunes at a distance of 800 metres from the coast at Freshfield, Lancashire (National Grid Reference: SD289096. The formation of dunes in this area began in the 17th century when sand was deposited over a layer of peat. The mineralogy of the sand suggests that it originated from the Millstone grits of the Pennines (and the boulder clays derived from these grits) and was carried to the coast by the River Mersey and the River Dee (Ashton, 1920).

Before afforestation, the dunes bore a whole complex of higher plant communities, the plant cover ranging from nil in freshly eroded areas to dense in areas colonized by *Ammophila arenaria*, *Carex arenaria*,

Dicranum spp. and *Polytrichum* spp. The more stable areas were colonized by *Hippophae rhamnoides*, *Festuca rubra*, *Carex arenaria*, *Lotus corniculatus*, *Viola canina*, *Hieracium pilosella* and *Fragaria vesca*. In the moister parts, the vegetation was dominated by *Salix repens*. Afforestation of the sampling area occurred about 1924. As a result, herbaceous plants have been almost completely eliminated; now only *Epipactis dunensis* and *Epipactis pendula* occur sporadically. At the present time the circumference of the pine trees, measured 1 metre above ground level, varies from 15 to 70 cm. The distance between individual trees is from 1.2 to 5.0 m.

The development of a dense carpet of pine needles has caused the formation of a distinct soil profile in an otherwise little stratified dune soil. The pine needles form a well marked A_0 horizon which overlies the mineral soil. L, F_1 , F_2 and H layers are all present, though the H layer is usually only a few millimetres thick. As the pine litter has been broken down, the decomposition products have been washed into the mineral soil and have caused the formation of an undifferentiated acidic A_1 horizon. This can be distinguished visually from the alkaline C horizon (parent dune sand), by its grey colour. This colour decreases in intensity and at a depth of 6 cm. it has usually disappeared, although

TABLE 1

Physical and chemical properties of the Freshfield pine forest soil

	A_1 horizon	C horizon
pH (glass electrode)*	3.7-4.9	7.6-8.8
Total Nitrogen (Kjeldahl method)**	210 ppm.	75 ppm.
% organic carbon***	0.67	0.17
% organic matter (loss on ignition, corrected for losses from carbonates)*	1.6	0.54
C/N ratio*	32.0	23.0
Chloride concentration (%)****	0.007	0.002
% free carbonate	0.0	2.1
Field capacity (%)*	30.0	24.2
Mechanical analysis*		
% coarse sand	46.3	41.9
% fine sand	50.5	55.4
% silt	0.18	0.19
% clay	0.22	0.58

* Piper (1944).

** Jackson (1958).

*** Shaw (1959).

**** Hall (pers. comm.)

it may extend to a depth of 14 cm. in a few places. In some places, a narrow but distinct B horizon is present, as judged by the deposition of iron at the A₁/C boundary. The C horizon consists of light yellow, *alkaline* sand, having a free calcim carbonate content of about 2 per cent. In contrast to the organic matter in the A horizons, which consists mainly of the products of decomposition and humification of leaf material, the organic matter in the C horizon originates chiefly from the roots of *Pinus*. An analysis of some of the physical and chemical properties of the soil is given in Table 1.

METHODS

Dilution plate technique

Soil was taken from the profile in sterile 3 in. × 1 in. glass tubes. Normally the soil that was sampled was taken from the middle of the A₁ horizon and from about 8 cm. below the A₁/C borderline in the C horizon. The initial dilution of the soil was prepared within 2 to 3 hrs. of sampling by adding 5.0 g. soil to 500 ml. $\frac{1}{4}$ strength Ringer's solution. The 10⁻² dilution obtained was agitated, using a magnetic stirring bar revolving at $2,800 \pm 40$ revs. per min. After agitating the suspension for 30 mins., 1.0 ml. was pipetted into 9.0 ml. of $\frac{1}{4}$ strength Ringer's solution containing 1,111 μ g. nystatin per ml. Using a fresh pipette, 1.0 ml. samples of this 10⁻³ dilution were transferred to each of 8 sterile Petri dishes containing a basal layer of 10 ml. sterile demineralized water agar (Orcutt, 1940). Twenty ml. peptone yeast extract actidione agar [0.5 per cent peptone; 0.1 per cent yeast extract (Oxoid); 0.001 per cent ferric phosphate; 0.005 per cent actidione; pH 7.0] were poured into each dish. The dishes were then rotated to ensure thorough mixing of the inoculum with the agar which was allowed to set. A further 1.0 ml. of the 10⁻³ dilution was then pipetted into another 9.0 ml. $\frac{1}{4}$ strength Ringer's solution containing 1,000 μ g. nystatin per ml. to obtain a 10⁻⁴ dilution. Agar plates were prepared from this dilution and further 10⁻⁵ and 10⁻⁶ dilutions prepared and plated when necessary. The plates were incubated at 25° for 14 days. They were placed upside down in vertical piles of 6 (Wilson, 1935). After rejecting plates with fungal and spreading bacterial colonies, colony counts were made to determine the numbers of bacteria present in the soil; alternatively bacteria were isolated for purification and identification. The results were expressed as 'bacteria per g. of oven dried soil, estimated on peptone yeast extract agar'. Only plates with between 30 and 300 colonies per plate were used in calculating the results (Breed and Dotterer, 1916; Wilson, 1935). In some

experiments soil extract agar was used, made from fertile garden soil (James, 1958).

In addition to the 50 μ g. actidione per ml., the medium used also contained 50 μ g. nystatin per ml., which originated from the dilution fluid. Nystatin was added in this way as it was thermolabile. Together these two antibiotics suppressed the growth of most of the fungi that would otherwise have interfered with the development of bacterial colonies (Williams and Davies, 1965).

All pipetting operations were preceded by sucking in and blowing out the suspensions 12 times in order to counteract the effects of adsorption of bacteria on the walls of the pipette (Wieringa, 1958).

Purification of isolates

All the bacteria growing on plates with 60 or fewer colonies were sub-cultured into tubes of peptone yeast broth (pH, 7.2) and grown for 7 days at 25°. These isolates were purified using the poured plate method. Discrete colonies were removed from the poured plates and purified further using the same technique once or twice more. The cultures obtained were maintained on peptone yeast extract agar at 5° under oil (Rhodes, 1957).

Morphological characters

These were recorded from bacteria that had been grown on nutrient agar slopes for 24 to 48 hrs. at 25°. Hucker's modification of Gram's method was used to stain the bacteria (Hucker and Conn, 1923). Motility was determined using the hanging drop method and the flagella were stained using Leifson's method (Leifson, 1951). Spore production was examined on 3 different media, peptone yeast extract agar, glucose nutrient agar and soil extract agar (Gordon and Ryneerson, 1964).

Colony characters

The characteristics of the bacterial colonies, e.g. shape, pigmentation, etc., were recorded on peptone yeast extract agar after 7 days incubation at 25°.

Growth and biochemical tests

These tests were performed mainly by using the multipoint inoculation method described by Goodfellow and Gray (1966) and Gray and Goodfellow (1966). All inoculations were made with bacteria from 18 to 24 hr. cultures in nutrient broth that had been growing at 25°. The inoculated test media were incubated for 3 days at 25° except

where specified otherwise. The tests used were as follows: growth in nutrient broth at different temperatures; growth in nutrient broth at different sodium chloride concentrations; acid production from sugars, sugar alcohols, alcohols and glycosides (Hugh and Leifson, 1953), using Seitz filtered or diethyl ether sterilized carbohydrates, etc.; catalase production on peptone yeast agar; casein and gelatin hydrolysis after 4 days (Smith, 1946); aesculin hydrolysis after 4 days, (Sneath, 1956); starch hydrolysis (Conn, Jennison and Weeks, 1957); tributyrin hydrolysis after 7 days, using Oxoid tributyrin agar; urease production (Christensen, 1946) (N.B. precautions were taken to ensure that the ammonia liberated from the medium in this test did not affect the medium in adjacent compartments in the dish by removing cultures from the dish as soon as they became positive); nitrate reduction to nitrite (Conn, Jennison and Weeks, 1957).

Other tests, especially those involving the production of gas or the clearing of insoluble compounds, were performed by more conventional methods for the reasons reported by Goodfellow and Gray (1966). The tests performed in this way were as follows: utilization of glucose by the oxidative or fermentative pathways after 7 and 14 days (Hugh and Leifson, 1953); hydrogen sulphide production from tryptophan using lead acetate papers (Conn, Jennison and Weeks, 1957); indole production from tryptophan (Kovács, 1928) after 2, 7 and 14 days; pectinase production after 7 days (Paton, 1959*a*); oxidase production (Steel, 1961); xylanase production (Sørensen, 1957); cellulose hydrolysis

TABLE 2

The occurrence of bacteria of different morphological types in the A₁ and C horizons

	Total	Morphological groups					
		1	2	3	4	5	6
Number of isolates—A ₁ horizon	259	11	24	154	7	36	27
Number of isolates—C horizon	268	24	74	63	34	26	47
% of isolates—A ₁ horizon	100	4	9	60	3	14	10
% of isolates—C horizon	100	9	27	24	13	10	17

1—Gram negative, pigmented rods.

2—Gram negative, non-pigmented rods.

3—Gram positive and Gram variable, spore-forming rods.

4—Gram positive streptomycetes.

5—Gram positive, pleomorphic rods.

6—Gram positive cocci and coccoid rods.

TABLE 3

*Selected biochemical characteristics of the morphological groups of bacteria
(percentage of strains positive) from the C horizon*

	Group 1 24 strains	Group 2 74 strains	Group 3 63 strains	Group 4 34 strains	Group 5 26 strains	Group 6 47 strains	Total 268 strains
Hydrolysis of starch	25	19	39	29	19	13	28
Liquefaction of pectin	—	7	19	—	12	4	8
Breakdown of xylan	4	5	25	14	—	4	9
Breakdown of cellulose	—	—	—	3	—	—	0.5
Hydrolysis of aesculin	42	42	73	56	16	34	43
Clearing of humic acid	—	—	5	53	4	—	9
Reduction of NO ₃ to NO ₂	12	15	57	38	46	47	32
Hydrolysis of gelatin	25	23	71	18	4	11	26
Hydrolysis of casein	17	5	71	12	31	30	30
Peptonization of purple milk	8	11	44	—	16	17	15
Urease production	4	7	2	—	23	4	6
Production of indole	—	—	—	—	—	—	—
Production of H ₂ S from peptone (7 days)	4	23	27	12	27	17	18
Production of H ₂ S from peptone (14 days)	12	31	29	18	35	30	29
Hydrolysis of tributyrin	12	39	60	18	42	40	40
Oxidase production	25	67	29	—	8	—	29
Catalase production (weak)	54	53	41	65	35	21	43
Catalase production (strong)	46	47	59	35	65	79	57
Growth in 3% NaCl	62	62	88	56	92	83	76
5% NaCl	42	59	85	41	81	81	69
7% NaCl	12	34	66	14	12	66	45
10% NaCl	8	23	47	6	8	58	31
15% NaCl	—	—	—	—	—	4	1
Growth at 30°	58	47	100	62	89	85	74
35°	42	32	100	32	38	66	57
40°	8	13	100	14	27	30	38
45°	8	4	79	9	8	19	25

TABLE 4

The fermentation patterns of the different morphological groups of bacteria (percentage of strains positive) from the C horizon

	Group 1 24 strains	Group 2 74 strains	Group 3 63 strains	Group 4 34 strains	Group 5 26 strains	Group 6 47 strains	Total 268 strains
Acid from glucose (Oxidative) 7 days	12	21	9	44	42	32	26
Acid from glucose (Oxidative) 14 days	33	34	17	68	50	47	41
Acid from glucose (Fermentative) 7 days	—	—	63	—	—	30	21
Acid from D-arabinose	—	18	8	9	12	13	12
L-arabinose	8	43	60	12	19	36	37
xylose	12	28	44	6	27	55	33
rhamnose	4	35	21	9	23	32	24
fructose	12	40	63	14	46	60	45
galactose	4	40	33	9	31	32	30
mannose	17	36	58	6	27	51	38
cellobiose	12	39	63	26	35	47	42
lactose	—	13	6	9	4	13	9

maltose	21	42	63	14	35	68	36
sucrose	12	38	65	9	38	60	43
trehalose	8	32	60	14	31	47	38
melizitose	4	32	15	9	19	45	24
raffinose	4	30	66	9	19	32	30
glycogen	—	—	27	3	—	9	9
inulin	12	28	68	—	27	40	31
amygdalin	12	32	55	12	27	47	36
salicin	8	24	66	6	27	36	27
dulcitol	4	11	2	3	12	26	10
glycerol	—	40	66	14	46	58	38
inositol	4	30	66	3	38	26	30
mannitol	8	38	76	14	27	50	40
sorbitol	4	36	47	6	42	49	36
glucosamine	4	16	65	3	23	43	30
dextrin	8	11	27	—	8	6	12
ethanol	—	24	13	12	27	29	16

N.B. Group 2 strains often appeared to attack many sugars and yet could not attack glucose. This is due to the fact that the attack of glucose was tested for by the original Hugh and Leifson (1953) technique, whilst the other tests were carried out using multipoint inoculation micro-techniques which are more sensitive.

(Eggins and Pugh, 1962), but in a medium at pH 7.2 and with 0.5 per cent cellulose read after 6 weeks; clearing of humic acid at pH 7.0 (Hurst, Burges and Latter, 1962), using humic acid supplied by L. Light and Co. Ltd. and reading the results during a 6 week period; action on purple milk after 7, 14 and 21 days; production of a fluorescent pigment (Paton, 1959*b*).

RESULTS

Characteristics possessed by the isolates

Using the tests described, 268 isolates from the C horizon soil were examined. Currently, a similar number of isolates from the A₁ horizon are being studied. The organisms could be divided into 6 morphological groups: (1) Gram-negative pigmented rods; (2) Gram-negative non-pigmented rods; (3) Gram-positive or Gram-variable spore-forming rods; (4) streptomycetes; (5) Gram-variable pleomorphic forms; (6) Gram-positive cocci and coccoid rods. Strains possessing diffusible fluorescent pigments were included in Group 2. The number and percentage of isolates in each group are shown in Table 2, whilst the results of the biochemical and growth tests are shown in Tables 3 and 4.

TABLE 5

The genera of bacteria present in the C horizon soil

Genus	Number of isolates	% of isolates
<i>Achromobacter</i>	32	12
<i>Arthrobacter</i>	21	8
<i>Bacillus</i>	63	24
<i>Flavobacterium</i>	24	9
<i>Micrococcus</i>	18	6
<i>Nocardia</i>	5	2
<i>Pseudomonas</i> (Group 1)*	5	2
(Group 2)*	11	4
(Group 3)*	24	9
<i>Staphylococcus</i>	17	6
<i>Streptomyces</i>	34	13
Unidentified	14	5

* *Pseudomonas*, Group 1: Fluorescent pigment; oxidative glucose dissimilation; gelatin hydrolysed; starch not hydrolysed.

Group 2: No pigment; oxidative glucose dissimilation; gelatin not hydrolysed; starch sometimes hydrolysed.

Group 3: No pigment; glucose not attacked; gelatin hydrolysed; starch not hydrolysed.

Generic composition of the bacterial flora

The Gram-negative rods were identified using a scheme based on that described by Shewan, Hobbs and Hodgkiss (1960). Spore-forming bacteria were determined using the key of Smith, Gordon and Clark (1952). Other forms were identified using Skerman's key (Skerman, 1959). The number and percentage of the isolates in each genus are shown in Table 5. In addition the species composition of the *Bacillus* populations in

TABLE 6

The species of Bacillus in the A₁ and C horizon soils

Species	% of isolates	
	A ₁ (pasteurized)	C (unpasteurized)
<i>B. licheniformis</i>	2.0	—
<i>B. subtilis</i>	13.0	59.0
<i>B. pumilus</i>	1.0	5.0
<i>B. firmus</i>	—	9.0
<i>B. lentus</i>	1.0	—
<i>B. alvei</i>	1.0	—
<i>B. circulans</i>	27.5	—
<i>B. circulans-laterosporus-brevis intermediates</i> (do not hydrolyse starch)	—	24.0
<i>B. brevis</i>	6.0	—
<i>Bacillus</i> (Group 1)*	—	3.0
Rods with spore-like swellings	32.5	—
Rods with no swellings and no spores	16.0	—

* Smith, Gordon and Clark (1953).

TABLE 7

The ability of Bacillus spp. to utilize ammonium nitrogen without growth factors

Species	No. of strains tested	No. of strains growing
<i>B. subtilis</i>	56	50
<i>B. licheniformis</i>	3	3
<i>B. pumilus</i>	3	1
<i>B. firmus</i>	5	0
<i>B. lentus</i>	3	1
<i>B. brevis</i>	9	0
<i>B. alvei</i>	2	0
<i>B. laterosporus</i>	1	0
<i>B. circulans</i>	56	3

TABLE 8
Variations in the numbers of bacteria and certain environmental factors in soil from the A₁ horizon

	Date of sampling										
	1963					1964					
	7/11	13/11	19/11	27/11	29/11	14/1	21/1	24/1	11/2	14/2	27/2
No. of bacteria/g. soil in millions	0.213	0.814	0.691	2.308	0.463	0.253	1.343	0.862	0.371	0.883	0.289
pH	3.8	4.4	3.7	4.0	3.9	4.3	3.9	4.2	4.0	4.0	3.8
Soil temperature	10.5	8.5	9.0	8.0	7.5	3.5	5.0	6.0	6.0	7.0	7.5
% moisture content	6.3	5.3	5.4	4.5	5.9	2.8	3.5	3.8	3.9	3.9	4.4
% organic carbon	0.6	0.6	0.6	0.4	0.6	0.5	0.6	0.7	0.6	0.5	0.6

both the A₁ and the C horizons are compared in Table 6. The bacilli from the A₁ horizon were isolated from dilution plates prepared from a soil suspension heated at 80° for 10 minutes. Table 7 shows the number of bacilli capable of utilizing ammonium nitrogen without added growth factors.

Numbers of bacteria in the soil

The results of a series of dilution plate counts of bacteria in the A₁ horizon soil, made over a 5 month period, are recorded in Table 8 together with variations in pH, soil temperature, percentage moisture content and organic carbon content. Table 9 compares counts of bacteria in samples taken from the A₁ and the C horizons. No correlations between any one factor and the numbers of bacteria in the soil are evident. This may mean that the counting methods employed are not sufficiently sensitive to detect correlations or that the variations in the

TABLE 9

Numbers of bacteria developing from A₁ and C horizon soils on different media

	Numbers of bacteria in millions/g. of soil			
	November, 1963		December, 1963	
	A ₁	C	A ₁	C
Peptone yeast extract agar pH 7.0	8.0	1.1	135.6 141.6 264.0	187.6 338.6 398.8
Peptone yeast extract agar pH 5.5	7.7	1.2	155.0 157.0	191.1 393.8
Garden soil extract agar pH 7.0	7.9	0.7	137.5 159.1	228.8 342.3

soil environment cause changes in the populations, other than quantitative ones. As a result, the figures are quoted merely to show the approximate size and variation of the population. Figures obtained by direct methods of counting give similar results and are referred to elsewhere in this symposium (pp. 173 and 185).

A feature of the counts worth noting is that a heated soil extract medium permitted the growth of smaller numbers of bacteria.

DISCUSSION

Different soil types have been shown to support characteristic bacterial floras. In cultivated soils, corynebacteria have been shown to be one of the dominant groups (Topping, 1937; Lochhead, 1939; Rouatt and Katznelson, 1961), although Gram-negative rods, e.g. *Pseudomonas* predominate in the rhizospheres of cultivated plants (Holding, 1960; Rouatt and Katznelson, 1961). In contrast, acidic, peaty soils are characterized by *Bacillus* species (Holding, Franklin and Watling, 1965). In this respect they resemble the acidic A₁ horizon soil of the present investigation, where bacilli constitute about 59.5 per cent of the isolates (cf. 67 per cent, Holding *et al.*, 1965).

In the alkaline C horizon soil the percentage of spore-forming bacteria is much reduced and the incidence of Gram-negative rods increased; cocci, streptomycetes and Gram-negative, pigmented rods are also more important. In some ways, this type of flora is similar to that reported by Jensen (1963) for a beech forest soil, where no one group of bacteria was clearly dominant. The increase in the number of streptomycetes in the alkaline soil agrees with the results of Jensen (1930); further, Taber (1960) found that streptomycetes from alkaline soils were partly suppressed by slight acidity. Evidently *Bacillus* species are much more tolerant of acid conditions, possibly because they are better fitted to survive in such environments as a result of their ability to produce endospores.

The commonest species of *Bacillus* in this soil are *B. subtilis*, *B. circulans* and forms closely related to, though not identical with, *B. circulans*. In addition there are a large number of forms isolated from the pasteurized samples which produce only spore-like swellings or no spores at all. Mishustin and Mirsoeva (p. 459) have suggested that the distribution of bacilli in the soil is related to their ability to use either organic or inorganic nitrogen. Thus in soils where mineralization is slow and there is little inorganic nitrogen, species with complex nutritional requirements will predominate. Examination of the bacilli isolated from the Freshfield soil for their ability to utilize ammonium nitrogen has shown that one of the common species does utilize inorganic nitrogen (*B. subtilis*), whilst the other does not (*B. circulans*). Though Mishustin and Mirsoeva do not deal with the distribution of *B. circulans*, it seems to us that their view may be an over-simplification. It does not account for the fact that organisms utilizing inorganic nitrogen can usually utilize organic nitrogen as well and yet are absent from soils abundant in organic nitrogen. Furthermore, as Proom and

Knight (1955) have shown, the ability of bacilli to utilize ammonium nitrogen is complicated by the fact that many strains will use ammonium nitrogen if growth factors are present.

An interesting feature of the results is the number of cocci isolated. These have been reported from other acid soils (Beck and Poschenrieder, 1958; Holding, Franklin and Watling, 1965). Generally, however, they are regarded as being insignificant (Conn, 1948; Stout, 1958; Jensen, 1963). The chloride concentration of the Freshfield soil is low, but is greater than that of a similar inland soil at Cannock Chase (Hall, personal communication). Possibly localized drying of the dune soils might lead to the occurrence of high salinities which would favour cocci.

Many workers have reported the occurrence of the genera *Achromobacter* and *Pseudomonas* in soil (Holding, 1960; Jensen, 1963; Stout, 1958). However, *Flavobacterium*, which is abundant in the dune soil, has previously been reported to be rare, although it is apparently common on plant leaves, including *Pinus* leaves (Stout, 1960*b*, 1961).

Most of the isolates proved to be inactive as far as their ability to attack complex carbohydrates was concerned, a feature also noted by Holding *et al.* (1965). Lignin was not attacked at all. Decomposition of xylan was restricted to a few strains of *Bacillus subtilis* and some streptomycetes. Cellulose was attacked only by 1 streptomycete isolate. Pectin was liquefied by a few strains from a wide range of types. In contrast, over half the streptomycetes caused decolourization of humic acid. Possibly these strains are not breaking the molecule down but are accumulating it on the cell surface (Flaig and Schmidt, 1957; Burges and Latter, 1960).

Some organisms seemed to be inactive in respect of almost all the biochemical reactions tested. These included the *Flavobacterium* spp. (Group 1, Table 2), the *Bacillus circulans-laterosporus-brevis* group, the *Arthrobacter* group and the *Achromobacter* group.

The schemes of identification used in this work are based on essentially monothetic classifications. We are attempting to classify the isolates we have obtained using polythetic methods. It will be interesting to see if these enable us to construct more satisfactory keys for the identification of the common forms of bacteria present in this soil.

ACKNOWLEDGMENTS

The authors wish to thank Mr. B. Hall of the Soil Survey for permission to quote some of his data and Mr. K. Winterhalder for determining the physical and chemical properties of the soil. One of us (I.R.H.) is

indebted to the Agricultural Research Council for financial support. We wish to thank Mr. R. Grierson and Miss A. P. Kerrigan for valuable technical assistance.

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